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Cytokine Measurement in Biological Samples After Physicochemical Treatment for Inactivation of Biosafety Level 4 Viral Agents

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Physicochemical techniques such as γ-irradiation, membrane disruption by detergents like sodium dodecyl sulfate (SDS), and fixation with formaldehyde or paraformaldehyde are routinely used to inactivate biological specimens from patients and animals infected with Filoviruses and Arena viruses that must be studied in BSL 4 facilities. The effects of these inactivation techniques on the levels of immunologically active proteins like cytokines and chemokines are not known. Therefore, we investigated the effect of several decontamination techniques on the immunoreactivity and bioactivity of the inflammatory cytokines IL-1β, IL-6, TNF-α, IL-12, and IFN-γ, the anti-inflammatory cytokine IL-10, and the chemokine IL-8 in biological specimens. SDS (96%–100% reduction), paraformaldehyde treatment (11%–100% reduction), and heat denaturation (75%–100% reduction) were found to decrease markedly the levels of all cytokines and chemokines as measured by enzyme-linked immunosorbent assays. In contrast, γ-irradiation was found to have little or no effect on the immunoreactivity of these cytokines/chemokines and on the biological activity of tumor necrosis factor (TNF) α. Our data suggest that, of the agents tested, γ-irradiation is the preferred technique for inactivation of biological specimens containing viral agents that require the use of BSL 4 for immunological studies. ***J. Med. Virol. 59:341–345, 1999.*** Published 1999 Wiley-Liss, Inc.†

KEY WORDS: gamma radiation; chemokines; viral inactivation

INTRODUCTION

Recent outbreaks of diseases caused by newly discovered and reemerging viruses worldwide have led to a heightened interest in the study of the pathogenesis of biosafety level 4 (BSL4) viruses, such as Ebola and Lassa fever viruses [Feldmann et al., 1996; Peters, 1996]. Although these agents are among the more readily inactivated viruses, the serious personal and public health risks associated with infections caused by these pathogens have made it difficult to conduct much-needed immunological and therapeutic research on viral hemorrhagic fevers (VHF). Immunological studies of infected humans or animal models of infections with the agents of VHF have been limited by the requirement of BSL4 laboratories because of safety considerations. A number of methods have been used successfully to inactivate RNA and DNA viruses, including treatment with γ-irradiation [Lupton, 1981; Mitchell and McCormick, 1984], UV light [Chepurnov et al., 1995], β-propiolactone [Budowsky and Zalesskaya, 1991], paraformaldehyde (PFA), sodium dodecyl sulfate (SDS), and heat denaturation [Mitchell and McCormick, 1984]. Most of these techniques have been demonstrated to inactivate viruses effectively and not to affect a number of blood and serum components [Elliott et al., 1982; Mitchell and McCormick, 1984] and nonviral protein antigens [Loutfy et al., 1998].

However, few data are available on how these treatments affect the biological activity and immunoreactivity of immune mediators, such as cytokines and chemokines. This is an important issue in studies of immune responses to viral antigens, since secretion of cellular cytokine and chemokines are commonly used as a surrogate for immune activation and cellular immunity [Doherty and Ahmed, 1997]. Indeed, recent studies in patients with Ebola have shown elevated levels of IFN-γ TNF-α, and IL-10 in acutely ill patients with viral antigenemia [Villinger et al., 1999] and suggested that some cytokines, interferon (IFN) γ in particular, may play an important role in viral clearance [Baize et al., 1999; Nabel, 1999].

In this study we have investigated the effect of some

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commonly used inactivation methods on the immunoreactivity and biological activity of cytokines in biological fluids. We studied both recombinant cytokines and chemokines added to culture media and endogenously secreted cytokines and chemokines in supernatants of mitogen-stimulated human peripheral blood mononuclear cells (PBMC) to determine the effect of physicochemical decontamination techniques on the purified cytokines and on cytokines in biological samples. Inactivation of BSL 4 viruses in biological samples from infected patients and animals makes it possible to analyze samples more conveniently in noncontainment (BSL 3 and lower) laboratories. We found that γ-irradiation provides the best technique for inactivation of infectious virus without altering the immunoreactivity of most cytokines and chemokines and the biological activity of TNF-α.

MATERIALS AND METHODS

Recombinant Cytokines and Chemokines

The following recombinant human cytokines were obtained from commercial suppliers: recombinant human interleukin (rhIL)-1β (Endogen, Woburn, MA), rhIL-6 (Pharmingen, San Diego, CA), rhIL-8 (Pharmingen), rhIL-10 (Pharmingen), rhIL-12 (R&D Scientific, CA), IFN-γ (Endogen), and TNF-α (R&D Scientific).

Cell Culture and Stimulation

PBMC were separated by differential gradient centrifugation on Ficoll-Hypaque (Organon Technika, Durham, NC), using standard techniques on blood drawn from three healthy volunteers after obtaining informed consent. Cells were cultured in complete medium, consisting of RPMI 1640 (Bio-Whittaker, Walkersville, MD) with 10% fetal calf serum (Hyclone, Logan, UT), glutamine (1 mM), HEPES (1 mM), and gentamicin (50 μg/ml; Life Technologies, Rockville, MD). Culture conditions included medium with no additive (medium control), or with 5-μg/ml phytohemagglutinin (PHA; Life Technologies) or 5 μg/ml lipopolysaccharide (LPS; Sigma, MO) for 48 hr before collection of cell supernatants. Supernatants were stored at –70°C until use in cytokine/chemokine assays.

Physicochemical Techniques for Viral Inactivation

Samples were treated with one of the following inactivation procedures: buffered PFA (Sigma, St. Louis, MO) prepared by heating phosphate-buffered saline, pH 7.4 to 70°C to dissolve the PFA and used at a final concentration of 2% or 0.2%; SDS (Life Technologies/GIBCO, Gaithersberg, MD) at a final concentration of 2% or 0.2%; heat (70°C for 30 or 60 min, or boiling for 5 min); and γ-irradiation (2×10^4 or 5×10^4 Gy). These techniques have been shown to inactivate virus infectivity to differing degrees for several BSL 4 viral pathogens [Ginoza, 1968; Elliott et al., 1982; Mitchell and McCormick, 1984]. Chemical treatment of samples with buffered PFA (0.2% or 2%) and SDS (0.2% or 2%) was performed by adding the reagents to the sample before incubation with capture antibodies. Untreated recombinant cytokines/chemokines or untreated samples were used in all experiments as controls.

Gamma Irradiation

Twenty thousand or 50,000 Gray (Gy, defined as 1-joule/Kg absorbed radiation dose) of Co^{60} high-energy γ-radiation was delivered via a Gammacell 220 (Atomic Energy of Canada, Ottawa, ON, Canada) in ~70 or ~170 min, respectively. Before irradiation, samples were stored at –70°C and dry ice was used for cooling during irradiation.

Cytokine Immunoassays

Cytokine levels were assayed by quantitative capture enzyme-linked immunosorbent assays (ELISAs) in untreated or inactivated samples according to the manufacturer's directions, using the capture/detection antibody pairs for IL-1β and IFN-γ (Endogen), IL-6, IL-8, IL-10, IL-12 p40 (Pharmingen) and TNF-α (R&D Systems). Captured cytokines were incubated for 1 hr with biotinylated antibodies and detected by a streptavidin-horse radish peroxidase conjugate (Amersham, Arlington Heights, IL) with ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for color development. Optical densities were read at 410 nm in a Dynatech Microplate reader (Dynatech, Chantilly, VA). Samples were assigned units (pg/ml) by using standard curves generated with recombinant cytokines as instructed by the manufacturer. The sensitivity of the assays ranged from 10 to 30 pg/ml. Each experiment was repeated at least three times.

TNF-α Bioassay

A bioassay for detection of TNF-α described previously [Hogan and Vogel, 1996] was used to quantitate bioactive TNF-α. Briefly, TNF-α–sensitive mouse fibroblast cells, L929/WEHI-13VAR (a gift of Dr. Francois Villinger), were grown to confluence in 96-well tissue culture plates in complete RPMI with glucose (4.5 g/L), sodium bicarbonate (1.5 mM), and sodium pyruvate (1 mM). After being washed with medium, samples were added in triplicate wells and diluted two-fold serially at least seven times. Recombinant cytokines were used to generate standard curves. Actinomycin D (2.7 μg/ml final; Life Technologies/GIBCO) was added to each well, and the plates were incubated for 16–18 hr at 37°C in 5% CO_2. TNF-α–induced cell death was determined by crystal violet staining (0.5% crystal violet in 20% ethanol; Sigma). Methanol (J.T. Baker, Phillipsburg, NJ), 200 μl/well, was added to the wells and the optical density at 570 nm was determined using an ELISA plate reader. Standard curves generated with recombinant TNF-α were employed to assign units of TNF-α activity in samples, defined as the concentration of TNF-α/β that lysed 50% of cells [Hogan and Vogel, 1996].

TABLE I. Effects of Physicochemical Treatment on Recombinant Human Cytokines in Biological Media

Treatment		Cytokine[a] IL-1β	IL-6	IL-8	IL-10	IL-12	IFN-γ	TNF-α
Boil	5 min	0	0	0	0	0	0	3.2
Heat (70°C)	30 mi	13	1	9	17	0	0	25
	60 min	0	0	10	7	0	0	10
γ-irradiation	2×10^4 Gy	86	116	101	85	97	100	109
	5×10^4 Gy	99	80	92	93	91	96	88
Paraformaldehyde	0.2%[b]	103	36	0	36	24	16	42
	2.0%[b]	89	0	0	1	2	0	12
SDS	0.2%[b]	0	0	0	0	0	0	4
	2.0%[b]	0	0	0	0	0	0	4

[a]Data expressed as a percentage of untreated control cytokine.
[b]Number represents final concentration of the reagent.

Statistical Analysis

Cytokine levels in treated samples were compared with untreated control levels either in pg/ml or converted to a percentage of the corresponding untreated sample to normalize the data.

RESULTS

Effect of Inactivation Techniques on Immunoreactivity of Recombinant Cytokines

We tested the effect of three approaches to virus inactivation on the detection of a number of biologically important cytokines and chemokines by using ELISAs. The cytokines selected for study represent a panel of inflammatory cytokines that play crucial roles in the inflammation associated with acute viral infections. As seen in Table I, the levels of detectable recombinant cytokines and chemokines in cell culture medium were significantly decreased after treatment with PFA (0%–100% decrease in 0.2% PFA and 11%–100% in 2% PFA), SDS (96%–100% reduction in 0.2% and 2% SDS), and heat denaturation (75%–100% decrease). γ-irradiation (2×10^4 or 5×10^4 Gy) did not alter the levels of these cytokines more than 20%, suggesting that viral inactivation with γ-irradiation may be the best technique if these cytokines are to be measured.

Effect of Inactivation Techniques on Cytokine Supernatants of Mitogen-Stimulated Peripheral Blood Mononuclear Cells

To further characterize the effect of virus inactivation on cytokine levels in biological samples, we tested the effect of physicochemical inactivation procedures on the levels of in vitro, mitogen-driven cytokines. Supernatants of PHA- or LPS-stimulated PBMC from healthy individuals were treated with γ-irradiation, PFA, SDS, or heat denaturation before being tested with ELISA for IL-1β, IL-6, IL-8, IL-10, or IFN-γ. As shown in Figure 1, we found that, as observed with the recombinant cytokine standards, all the inactivation techniques except γ-irradiation decreased the levels of cytokines/chemokines detected by 40%–100%. LPS-stimulated IFN-γ and IL-1 were found to be reduced by γ-irradiation in both individuals studied (Fig. 1). One explanation for this observation is that LPS may sensitize these proteins (but not the others studied) to the damaging effects of γ-rays. However, PHA-stimulated IFN-γ and IL-1β were not reduced to the same extent by γ-irradiation, suggesting that sensitivity to γ-radiation is influenced by several environmental param-

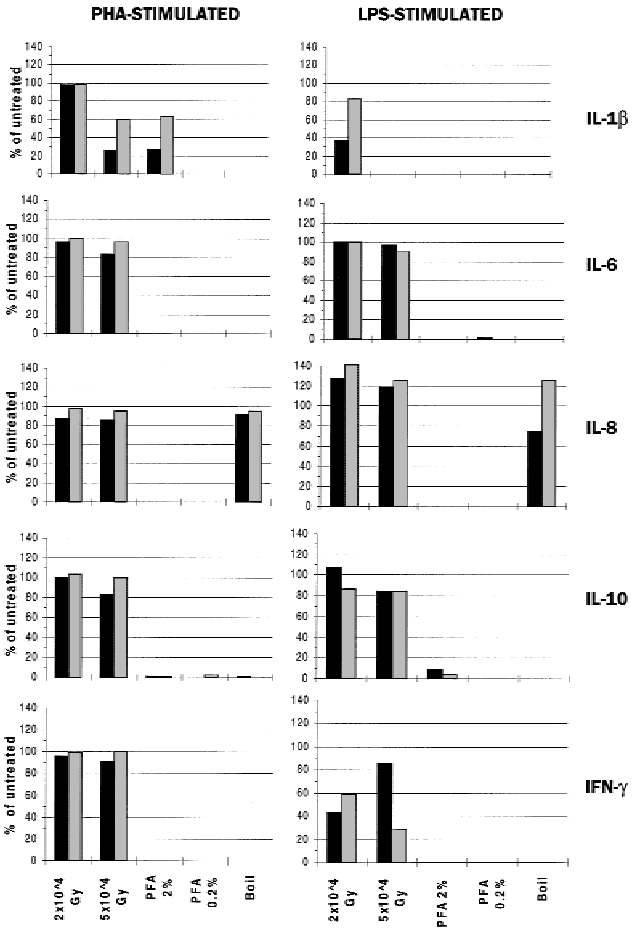

Fig. 1. Effects of physicochemical viral inactivation methods on the immunoreactivity of mitogen-driven cytokine and chemokines. Levels of indicated cytokines (IL-1β, IL-6, IL-8, IL-10, and IFN-γ) in supernatants of 5×10^6 PBMC from two healthy individuals (represented by the gray and black bars in each graph) incubated with PHA (5 μg/ml; left-side graphs) or LPS (5 μg/ml; right-side graphs) following treatment as indicated on the abscissae.

eters. Of note is that the apparent inverse relationship between dose of γ-irradiation and LPS-stimulated IFN-γ (Fig. 1) was not found consistently in repeat experiments (data not shown).

Another interesting finding with PBMC supernatants was that IL-8 was unaffected by heat denaturation (boiling). This result contrasted with our data from treatment of rIL-8. This suggests that native IL-8 may be more resistant to heat denaturation than rIL-8, perhaps because of differences in glycosylation or other posttranslational changes in the protein.

Effect of γ-Irradiation on Biological Activity of TNF-α/β

Since ELISAs for cytokines do not provide information on the bioactivity of the proteins, we investigated the effect of virus inactivation on biological activity of TNF-α by using a sensitive bioassay [Hogan and Vogel, 1996]. Only γ-irradiation was tested as an inactivation technique, because the results of the ELISA assays indicated that PFA, SDS, and heat treatment probably induced structural changes, and such changes are likely to have a profound impact on the bioactivity of proteins. As seen in Figure 2, γ-irradiation was found to result in a < 15% reduction of bioactivity of both recombinant and mitogen-stimulated native TNF-α. Taken together, these data suggest that γ-irradiation is the technique of choice for inactivating biological samples containing BSL 4 viruses if immunological responses need to be studied in biological samples.

DISCUSSION

The application of immunological assays to characterize cellular and humoral responses to viral antigens has presented unique problems in the study of BSL 4 viruses. When these agents are studied there is a need for virus inactivation without affecting the levels of immunological mediators in biological specimens. We evaluated the effects of viral inactivation methods on the levels of immunological mediators because of our interest in the investigation of cellular and humoral immunity to Ebola virus, and Lassa and related Arena viruses, all of which are BSL level 4 agents.

Our data demonstrate that, of the methods tested, γ-irradiation is the preferred method of inactivation because it least affects the levels of a number of cytokines and chemokines, when immunoassays (Fig. 1) or bioassays (Fig. 2) are used for detection. γ-irradiation has been shown to be an effective method for virus inactivation for many applications. The main disadvantage of its use is the lack of widespread access to Co^{60} irradiators. However, since specimens collected and stored in dry ice or liquid nitrogen are routinely transported to distant laboratories for diagnostic tests, biological specimen could similarly be transported to laboratories that do have access to Co^{60} irradiators for treatment and return to the original laboratory after inactivation for further analysis.

We found that chemical treatments, specifically PFA and SDS, altered cytokine and chemokine proteins sufficiently to compromise their detection by immunoassays. Other chemical methods that have been found not to alter proteins, e.g., β-propiolactone, are potentially carcinogenic [Joklik, 1980] and, in some situations, can alter the antigenicity of viral proteins [Mitchell and McCormick, 1984]. Other forms of ionizing radiation, e.g., UV light, while having the advantage of widespread availability and low cost, require considerable standardization in dose, wave length, temperature, and exposure time to ensure inactivation of all BSL 4

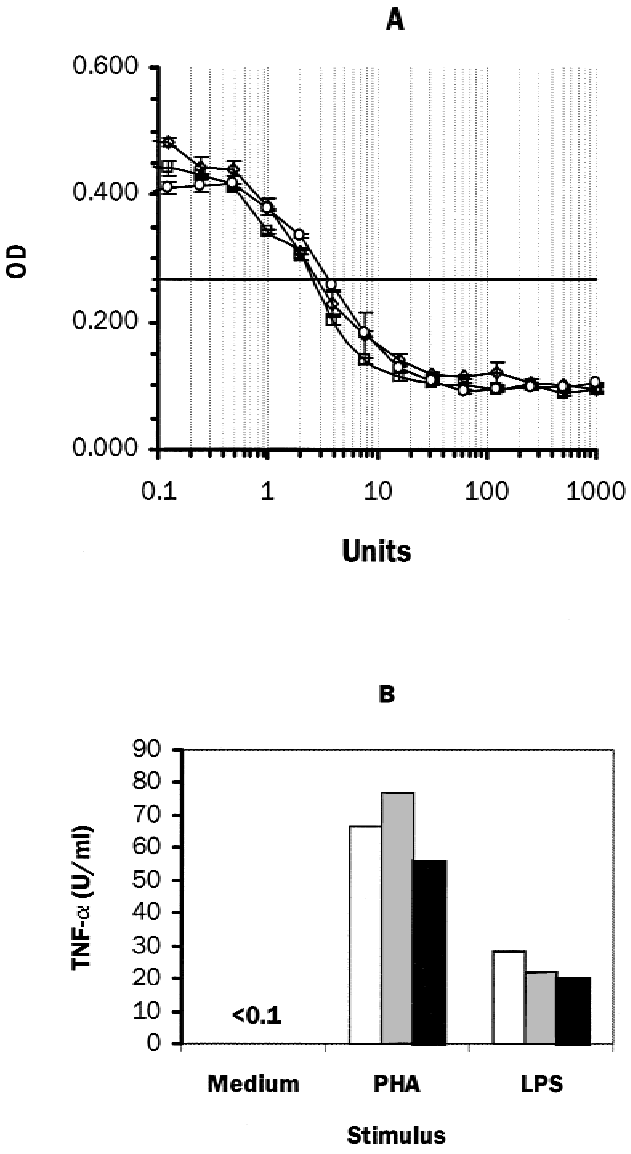

Fig. 2. Effects of γ-irradiation on the bioactivity of rhTNF-α and mitogen-stimulated TNF-α. **A:** Standard curves generated from serial twofold dilutions of untreated (squares) or γ-irradiated rhTNF-α with 2×10^4 Gy (diamonds) and 5×10^4 Gy (circles). Error bars represent standard errors of triplicate samples. The solid line indicates 50% cytotoxicity. **B:** TNF-α levels in supernatants of 5×10^6 PBMC from a healthy individual that were collected after a 48-hr incubation with no stimulus (medium), PHA (5 μg/ml), or LPS (5 μg/ml) following no γ-irradiation (unshaded bars), 2×10^4 Gy (gray bars), or 5×10^4 Gy (black bars). Representative data from three experiments are shown in graph.

viruses in different biological samples [Chepurnov et al., 1995]. Photochemical inactivation using psoralen derivatives, which acts selectively on the virus nucleic acid, has shown considerable promise in the inactivation of DNA and RNA viruses [Hanson et al., 1978]. However, the effect of these chemicals on immunologically active proteins in biological sample is not known [Margolis-Nunno et al., 1992].

As with other ionizing radiation, after absorption by tissue or biological fluids, γ-rays typically release a large amount of energy within a small space. The direct result of such localized action may be simple, such as breakage of a single covalent bond, which, if critical for macromolecular function, may interrupt the function of a given protein or nucleic acid molecule by inducing an overt physical change in structure. However, several factors influence the efficiency of γ-radiation–induced macromolecular damage. The length of DNA/RNA (viral genome size), concentration of serum in the buffer, and the temperature at which γ-irradiation occurs can all influence the ability of a given dose of γ-radiation to inactivate viruses. The salient advantage of this technique of inactivation is the lack of alteration of virus antigenic integrity as determined by indirect immunofluorescence, immunoassays, and reactivity with monoclonal antibodies [Elliott et al., 1982]. Our data extend these conclusions to nonviral proteins in biological specimens, specifically, to immunologic mediators in infected biological samples, demonstrating the lack of alteration of cytokines by γ-irradiation.

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